Gut colonization with methanobrevibacter smithii is associated with childhood weight development

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EPIDEMIOLOGY/GENETICS

Obesity

Gut Colonization with *Methanobrevibacter smithii* is Associated with Childhood Weight Development

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Objective: To prospectively investigate the presence and counts of archaea in feces of 472 children in association with weight development from 6 to 10 years of age.
Methods: Within the KOALA Birth Cohort Study, a single fecal sample from each child was analyzed by quantitative polymerase chain reaction to quantify archaea (*Methanobrevibacter smithii, Methanosphera stadtmanae*). Anthropometric outcomes (overweight [body mass index {BMI} $\geq$ 85th percentile], age- and sex-standardized BMI, weight, and height *z*-scores) were repeatedly measured at ages (mean $\pm$ SD) of 6.2 $\pm$ 0.5, 6.8 $\pm$ 0.5, 7.8 $\pm$ 0.5, and 8.8 $\pm$ 0.5 years. Generalized estimating equation was used for statistical analysis while controlling for confounders.
Results: *Methanobrevibacter smithii* colonization was associated with an increased risk of overweight (adjusted odds ratio [OR] = 2.69; 95% confidence interval [CI] 0.96-7.54) from 6 to 10 years of age. Children with high levels (>7 $\log_{10}$ copies/g feces) of this archaeon were at highest risk for overweight (OR = 3.27; 95% CI 1.09-9.83). Moreover, *M. smithii* colonization was associated with higher weight *z*-scores (adj. β 0.18; 95% CI 0.00-0.36), but not with height. For BMI *z*-scores, the interaction (P = 0.008) between *M. smithii* and age was statistically significant, implying children colonized with *M. smithii* had increasing BMI *z*-scores with age.
Conclusions: Presence and higher counts of *M. smithii* in the gut of children are associated with higher weight *z*-scores, higher BMI *z*-scores, and overweight.

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Introduction

Over the past decades, childhood overweight and obesity have reached epidemic proportions in most industrialized countries (1). Since 1980, there has been a two- to threefold increase in childhood overweight and four- to sixfold increase in obesity in the Netherlands (2). Eventually, children with overweight or obesity are more likely to become adults with obesity (3,4), leading to increased risk for chronic diseases, including cardiovascular disease, hypertension, type 2 diabetes

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mellitus, and premature mortality (2). Moreover, once obesity is established, it is difficult to reverse through interventions (5).

Recently, a role of the gut microbiota has been put forward. The human gut microbiota is a complex, densely populated ecosystem, mainly consisting of bacteria (6). Besides the many bacterial species, the human gut contains several archaeal species, which are considered to play a crucial role in the metabolic capacity of the human gut microbiota. To date, only three distinct species within the group of methanogenic archaea have been isolated from human feces, *Methanobrevibacter smithii* (7), *Methanospaera stadtmanae*, (8), and *Methanomassilicoccus luminyesis* (9). In children from 1 to 10 years of age, the prevalence of these methanogenic species have been found in 88%, 11%, and 1% of the children, respectively (10). Methanogens use hydrogen, a by-product of bacterial fermentation, to produce methane. The removal of hydrogen accelerates the bacterial fermentation of polysaccharides and carbohydrates, leading to the more efficient production of short-chain fatty acids which can serve as an additional energy source for the host (11-15).

Several studies, both in animal models and humans, have suggested a potential role for archaea, specifically *M. smithii,* in the development of overweight/obesity. Using a mouse model, Samuel et al. illustrated that colonization with *M. smithii* leads to increased utilization of dietary fiber and increased adiposity (16). Another study revealed a significant increase in *M. smithii* in mice fed with high-fat chow compared with those fed with normal chow (17). Only few studies have addressed the relationship between archaea and host energy balance in humans. In a small study including nine human participants, Zhang et al. (18) detected significantly higher numbers of H_2-utilizing methanogenic archaea in individuals with obesity compared with normal-weight as well as post-gastric-bypass individuals. In contrast, other studies have reported lower amounts of *M. smithii* in individuals with obesity compared with lean individuals (19,20). These results demonstrate that more research is needed to understand the role of archaea in obesity (20). Most of the studies reporting a positive association between methanogenic archaea and obesity were based on breath methane measurements. A study among 58 subjects with obesity showed a significantly higher body mass index (BMI) among breath methane-positive as compared with methane-negative subjects (21). Moreover, in a large study among 792 subjects, the presence of both hydrogen and methane on breath testing was associated with a higher BMI and percent body fat (22). Breath methane measurements alone may, however, underestimate the number of participants with methanogenic archaea due to the lower sensitivity (62%) as compared with molecular detection of methanogens in stools (23,24). Moreover, the main evidence originates from animal studies, and the limited number of human studies that used fecal samples had small sample sizes, hence low power to detect a significant association, and a cross-sectional design. Lastly, none of these studies has been performed in children.

Therefore, we aimed to investigate whether the presence and counts of archaea in the fecal samples of 472 children at school age are associated with childhood weight development from 6 to 10 years of age.

Methods

Study design

The present study was conducted within the prospective KOALA Birth Cohort Study, in the Netherlands. The design of this study has been described in detail elsewhere (25). A total of 2,834 pregnant women were recruited, at 34 weeks of gestation, from October 2000 until December 2002. Healthy pregnant women with a conventional lifestyle (n = 2,343) were retrieved from an on-going cohort study on the etiology of pregnancy-related pelvic girdle pain in the Netherlands. An additional 491 pregnant women with alternative lifestyles were recruited through organic food shops, anthroposophist doctors and midwives, Steiner schools, and dedicated magazines. This latter group of women was considered to have an alternative lifestyle that could involve dietary habits (vegetarian, organic food choice), child-rearing practices, vaccination schemes, and/or use of antibiotics. Exclusion criteria for the present study were: prematurity (<37 weeks of gestation), twins, abnormalities linked to growth (such as Down's syndrome, Turner syndrome, Fallot's tetralogy, multiple disabilities, and cystic fibrosis), and fecal samples whose transport time was greater than 4 days. All children included in this study were Caucasians. Informed consent was given by all parents, and the study was approved by the Medical Ethics Committee of Maastricht University and the National Ethical Committee for Medical Research.

Data collection and longitudinal outcome measures

At 14 and 34 weeks of gestation, pregnant women received questionnaires collecting data on amongst others family size, pre-pregnancy height and weight, and weight gain during pregnancy. Two weeks after childbirth, data was collected from obstetric reports and questionnaires were completed by the mothers to obtain data on gestational age, birth weight, and gender of the child. Food frequency questionnaires were filled out by the parents to report the dietary habits of their children at the age (mean ± standard deviation [SD]) of 5.0 ± 0.6 years.

Parent-reported weight and height of children was repeatedly assessed by mailing follow-up questionnaires to the parents in 2007, 2008, 2009, and 2010. This corresponds to longitudinal assessment of the children's height and weight at the ages (mean ± SD) of 6.2 ± 0.5, 6.8 ± 0.5, 7.8 ± 0.5, and 8.8 ± 0.5 years, respectively.

BMI was computed as weight divided by height squared (kg/m^2). The BMI, weight, and height measurements were then converted into age- and gender-specific z-scores using the Dutch National growth study (26) as the reference population. BMI z-scores were used both as continuous and dichotomous outcomes. Dichotomization into normal weight versus overweight was based upon a cut-off of a z-score ≥1.04, equivalent to BMI z-scores ≥85th percentile standardized for age and gender (27).

Fecal sample collection

A subgroup of this cohort (n = 1,204), that is, the participants who were visited at home for blood collection from the mother during pregnancy and/or the child at age 2 years, and were still active participants at child's age of 6-7 years (see flowchart, Figure 1), was asked to collect a single fecal sample from their children at school age. Feces collection tubes with spoon attached to their lids (Sarstedt, Nümbrecht, Germany) together with collection instructions were sent to the parents of participating children. A fecal sample was collected and sent to the laboratory by mail. Upon arrival in the laboratory, fecal samples were 10-fold diluted in peptone/water (Oxoid CM0009) containing 20% (vol/vol) glycerol (Merck, Darmstadt, Germany) and stored at −80°C until further analysis.

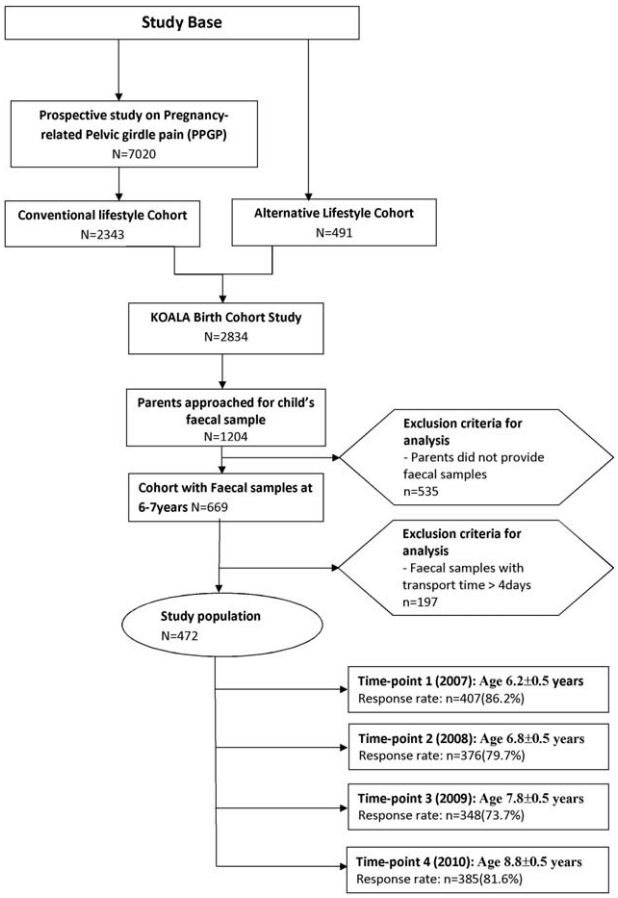

Figure 1 Flowchart illustrating how the present study population of 472 children was obtained from the initial KOALA cohort of 2,834 healthy pregnant women.

Fecal DNA isolation and real-time quantitative polymerase chain reaction

DNA isolation from fecal samples has been described in detail elsewhere (28). In brief, the DNA was isolated using a combination of Repeated-Bead-Beading (RBB) plus column purification method. Concentration and purity of the DNA were assessed with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington).

DNA from all fecal samples was subjected to 5′-nuclease based real-time polymerase chain reaction (PCR) assays for the enumeration of *M. smithii* and *M. stadtmanae* (primers and probes are listed in supplementary Table 1). For both *M. smithii* and *M. stadtmanae*, amplifications were conducted in a total volume of 25 μL, containing 1× Absolute quantitative (qPCR) Mix (ABgene, Hamburg, Germany), 200 nM of forward and reverse primers, 200 nM TaqMan probe, and 2 μL of tenfold diluted target DNA. The amplification (2 minutes at 50°C, 10 minutes at 95°C, and 42 cycles of 15 seconds at 95°C and 1 minute at 60°C) and detection were conducted with an Applied Biosystems Prism 7900 sequence detection system (Applied Biosystems). Quantification of *M. smithii* and *M. stadtmanae* was achieved by using a quantification plasmid containing the target sequences. Plasmid constructs containing the sequence of interest were created as positive controls (see Supporting Information S1 and Figure S1(a) and (b) for standard curves). The lower limits of detection were 3.81 and 4.82 $\log_{10}$ copies/g feces for *M. smithii* and *M. stadtmanae,* respectively. In the present study fecal samples were sent by mail and transport time varied from 0 to 4 days. However, the proportion of positive samples as well as the concentration of archaea did not significantly differ between samples according to transport time (Chi-square test, *P*-value >0.05).

Statistical analyses

Characteristics of the children are presented as mean ± SD or median (range) for continuous variables, and proportions for categorical variables. We used Generalized Estimating Equations (GEE) with autoregressive correlation structure to analyze the association between archaea and childhood BMI, weight, and height (as *z*-scores for continuous outcomes) and overweight status over time (as binary outcome) including all available parent-reported repeated measurements up to 10 years of age. For each of these outcomes we examined the effects of colonization with either *M. smithii* or *M. stadtmanae* (yes/no) and the counts of each of these species. For *M. smithii,* we constructed a variable accounting for the tri-modal distribution of the counts (uncolonized, low levels [3.5-7 $\log_{10}$ copies/g feces], high levels [>7 $\log_{10}$ copies/g feces]) observed (Figure 2). To evaluate whether increasing levels of the counts of *M. smithii* were associated with higher BMI *z*-scores, Cochran Armitage trend test was performed for this exposure–outcome combination.

To investigate whether results differed across ages, we tested for statistical interaction between the main independent variables and age of the child at the time of outcome measurement as a continuous variable. The interaction term was statistically significant only for *M. smithii* prevalence (yes/no) with BMI *z*-scores as the outcome

TABLE 1 Real-time PCR primers and probe sequences for detecting *M. smithii* and *M. stadtmanae* 16S rRNA[a]

Target organisms (amplicon size)	Primer/probe	Sequence (5′-3′)	T_m (°C)
M. smithii **(123 bp)**	Forward primer	5′-CCGGGTATCTAATCCGGTTC-3′	63.0
	Reverse primer	5′-CTCCCAGGGTAGAGGTGAAA-3′	64.0
	Probe	5′-CCGTCAGAATCGTTCCAGTCAG-3′	61.0
M. stadtmanae **(97 bp)**	Forward primer	5′-AGGAGCGACAGCAGAATGAT-3′	64.0
	Reverse primer	5′-CAGGACGCTTCACAGTACGA-3′	65.0
	Probe	5′-TGAGAGGAGGTGCATGGCCG-3′	71.0

[a]Reference for information in table, Dridi et al. (2009) (11).

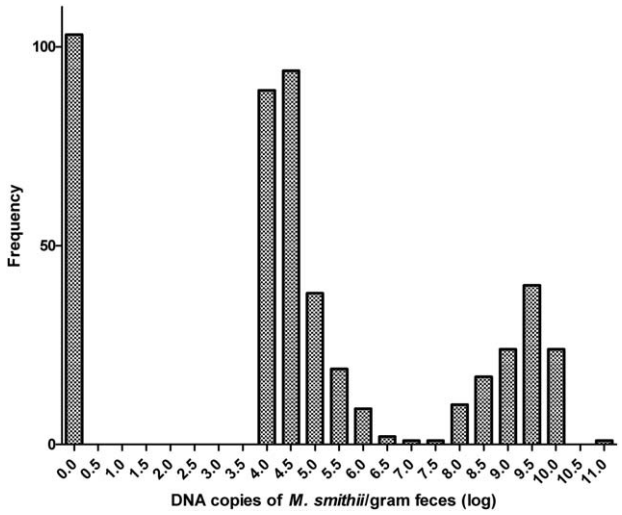

Figure 2 Histogram showing the trimodal distribution of counts (log_{10} DNA copies/g feces) for *M. smithii*.

(interaction $P = 0.008$); hence an age-stratified analysis was performed for this association.

Potential confounders considered for inclusion in the model were: recruitment group (conventional or alternative), maternal pre-pregnancy weight, maternal pre-pregnancy height, maternal educational level (lower education, vocational education, higher general secondary/pre-university, or higher vocational/academic education), weight gained during pregnancy, place and mode of delivery (vaginal delivery at home, vaginal delivery in hospital, or caesarean section in hospital), gestational age, birth weight, household size, antibiotic use (no antibiotics in past year, antibiotic use >4 weeks ago, or antibiotic use ≤4 weeks ago), physical activity, gender, and child's dietary intake (total fiber, total energy, fats, and carbohydrates both as percentage of total energy). Variables that changed parameter estimates of the main independent variable by more than 10% were included in the final models. All children ($N = 472$) were included in the unadjusted analyses and a total of 406 (86%) children was included in the adjusted analyses. The latter was due to missing values in some confounders, ranging from 1.3% missing values for mode of delivery and antibiotic use to 6.1% for physical activity. We checked whether results obtained following multiple imputations deviated from the results obtained without imputation. Multiple imputations were done using all four repeated measurements for BMI *z*-scores, weight *z*-scores, and height *z*-scores variables including confounders with missing values. As results from combined imputed datasets ($n = 10$) were comparable with those of the original non-imputed data, we performed the final analyses without imputation.

The following statistical software was used: SAS version 9.3 and SPSS version 21.0 (SPSS Inc., Chicago, IL). A pre-selected significance level of 0.05 was used.

Results

A total of 472 fecal samples were eligible for analysis (Figure 1). *M. smithii* was present in 369 (78.2%), and *M. stadtmanae* in 39 (8.3%) of the 472 children (Table 2). From all these children, at least one anthropometric measurement was available. At time point 1 (start of follow-up) anthropometric data were available for 407 out of the 472 children, and 6.9% (28/407) of the children were overweight (Table 3). At this same time point, the percentage overweight among children colonized with *M. smithii* was 7.6% compared with 4.4% for children not colonized with *M. smithii*. While the percentage overweight among children colonized with *M. stadtmanae*, was 6.2% compared with 6.9% among children not colonized with this archaeon. For the subsequent follow-up time points, anthropometric data were available for 376, 348, and 385 out of 472 children, respectively.

Unadjusted and adjusted associations between the presence, counts and levels of counts of *M. smithii and M. stadtmanae* and the different outcome variables are presented in Table 4 (for overweight as binary outcome) and Table 5 (for BMI, weight, and height *z*-scores as continuous outcomes). In the unadjusted analyses we found no statistically significant association between the presence/counts of archaea and overweight status. Upon adjusting for confounders, children that were colonized with *M. smithii* were at increased risk of being overweight (adjusted odds ratio [OR] = 2.69; 95% confidence interval [CI] 0.96-7.54). Children with a low level of counts (3.5-7 log_{10} copies/g feces) of *M. smithii* were twice more likely to be overweight compared with children without this archaeon, although this association was not significant ($OR_{adjusted} = 2.40$; 95% CI 0.83-6.95). Children having a high level of counts (>7 log_{10} copies/g feces) of *M. smithii* were three times more likely to be overweight ($OR_{adjusted} = 3.27$; 95% CI 1.09-9.83). *P* for trend across these levels (none, low, and high) of *M. smithii* and overweight status approached significance (Cochran Armitage $P = 0.066$). Analyses on the counts of *M. smithii* as a continuous variable also showed that, a log_{10} increase in the counts of *M. smithii* was associated with a 10% increased risk of overweight ($OR_{adjusted} = 1.10$; 95% CI 1.00-1.21). No statistically significant association between *M. stadtmanae,* neither for presence nor counts, and overweight status was found.

Regarding BMI *z*-score as a continuous outcome variable, we found a statistically significant interaction between the presence of *M. smithii* and age (*P*-value = 0.008), implying, colonization with *M. smithii* was associated with an increasing BMI *z*-score as age increased (Figure 3). However, differences at individual time (age) points after stratifying for age did not reach statistical significance (data not shown).

Unadjusted analyses for the associations between the presence and counts of *M. smithii* and *M. stadtmanae* with weight *z*-scores as a continuous variable were not statistically significant. In the adjusted analyses, the presence of *M. smithii* in children was statistically significantly associated with higher weight *z*-scores (adj. β 0.18; 95% CI 0.00-0.36) compared with children without this archaeon. In addition, low and high levels of *M. smithii* in children were also associated with higher weight *z*-scores compared with uncolonized children, although this only approached statistical significance (low: adj. β 0.18; 95% CI −0.01 to 0.36 and high: adj. β 0.20; 95% CI −0.01 to 0.40; Table 5). The *P* for trend across these levels (none, low, and high) of counts of *M. smithii* approached significance in association with increasing weight *z*-scores (*P* for trend = 0.09).

There was no statistically significant association of *M. smithii* with height *z*-scores.

TABLE 2 Participant characteristics of the study population at start of the follow-up

	Study population (N = 472)[a], mean ± SD	*M. smithii*		*M. stadtmanae*	
		"Present" (n = 369), mean ± SD	"Absent" (n = 103), mean ± SD	"Present" (n = 39), mean ± SD	"Absent" (n = 433), mean ± SD
Dietary factors					
Total energy intake (KJ)	6143.2 ± 1255.0	6131.6 ± 1223.3	6185.7 ± 1370.4	6318.9 ± 1402.9	6127.2 ± 1241.8
% Energy intake from fats	29.7 ± 4.2	29.6 ± 4.2	29.8 ± 4.0	29.5 ± 4.6	29.7 ± 4.1
% Energy intake from carbohydrates	56.7 ± 5.9	55.7 ± 4.9	55.5 ± 5.1	56.4 ± 5.3	55.6 ± 4.9
Total fiber intake (g)	15.5 ± 3.9	15.4 ± 3.9	15.9 ± 3.5	15.9 ± 3.9	15.4 ± 3.8
Total physical activity (hours/week)	9.4 ± 4.4	9.3 ± 4.4	9.7 ± 4.1	9.6 ± 4.7	9.3 ± 4.3
Total household size	4.3 ± 0.8	4.3 ± 0.7	4.4 ± 0.9	4.3 ± 0.8	4.3 ± 0.8
Birth weight (g)	3574 ± 483	3560 ± 481	3624 ± 487	3676 ± 498	3566 ± 481
Place and mode of delivery, n (%)					
Vaginal delivery at home	219 (47.0)	176 (48.6)	43 (41.8)	16 (41.0)	203 (47.6)
Vaginal delivery in the hospital	198 (42.6)	150 (41.5)	48 (46.6)	15 (38.5)	183 (43.0)
Caesarean section in the hospital	48 (10.4)	36 (9.9)	12 (11.6)	8 (20.5)	40 (9.4)
Time of last antibiotic course, n (%)[b]					
No antibiotic use	398 (85.4)	310 (85.2)	88 (86.2)	35 (89.7)	365 (85.0)
>4 weeks ago	57 (12.2)	46 (12.6)	11 (10.8)	4 (4.3)	53 (12.4)
<4 weeks ago	11 (2.4)	8 (2.2)	3 (3)	0 (0.0)	11 (2.6)
Archaea counts (log_{10} DNA copies/g feces), median [range][c]		4.7 (3.8-10.8)		5.4 (4.8-9.2)	

[a]Totals may not add up to 472 because of missing values (for number of missing, see "Results" section).
[b]Time of last antibiotic course at time of fecal collection.
[c]Median counts were calculated from archaea (*M. smithii* and/or *M. stadtmanae*) positive samples only.

For *M. stadtmanae*, there were no statistically significant associations with any of the four outcomes (Tables 4 and 5) neither for presence or the counts of *M. stadtmanae*.

Discussion

This prospective cohort study is the first to demonstrate that the presence as well as the counts of methanogenic archaea, specifically *M. smithii,* in the gut of children at school age is associated with more overweight and higher weight *z*-scores. We also found that the strength of the association between colonization with *M. smithii* and BMI *z*-scores increased with age from 6 to 10 years.

Several previous studies (16,18) supported the hypothesis that archaea (*M. smithii*) contribute to energy harvesting and hence weight development. Samuel and Gordon (2006) (16), observed that *M. smithii* played a critical role in facilitating an increased capacity of *Bacteroides thetaiotaomicron* to digest polyfructose-containing glycans leading to increased production of short-chain fatty acids (SCFAs) and total liver triglycerides in mice. Mice colonized with *B. thetaiotaomicron* and *Desulfovibrio piger* instead of *M. smithii*, did not show such an effect, highlighting the key role of *M. smithii* in

TABLE 3 Anthropometric measures of the study population at the four different follow-up time points

	Time point 1 (2007), mean ± SD	Time point 2 (2008), mean ± SD	Time point 3 (2009), mean ± SD	Time point 4 (2010), mean ± SD
Children with anthropometric data, n (%)	407 (86.2)	376 (79.7)	348 (73.7)	385 (81.6)
Age (years)	6.2 ± 0.5	6.8 ± 0.5	7.8± 0.5	8.8 ± 0.5
Overweight, n (%)				
Yes	28 (6.9)	22 (5.9)	31 (8.9)	41 (10.7)
No	379 (93.1)	354 (94.1)	317 (91.1)	344 (89.3)
BMI *z*-scores	−0.33 ± 0.95	−0.37 ± 0.90	−0.26 ± 0.97	−0.22 ± 0.96
Weight *z*-scores	−0.37 ± 0.95	−0.35 ± 0.96	−0.27 ± 0.98	−0.19 ± 0.91
Height *z*-scores	−0.05 ± 0.96	−0.10 ± 0.98	−0.09 ± 0.96	0.08 ± 0.90

TABLE 4 GEE results showing association of overweight (yes/no) from 6 to 10 years of age with the prevalence of colonization and counts (log_{10} DNA copies/g feces) of archaea species in the gut microbiota at 6-7 years of age[a]

	n	Crude OR [95% CI][b]	Adjusted OR [95% CI][c]	*P*-value[d]
OVERWEIGHT (YES/NO)				
M. smithii prevalence				
No	103	1.00 [reference]	1.00 [reference]	
Yes	369	1.75 [0.77-3.96]	2.69 [0.96-7.54]	0.059
M. smithii count levels				
None	103	1.00 [reference]	1.00 [reference]	
Low ($\leq$7 log_{10} DNA copies/g feces)	251	1.64 [0.69-3.86]	2.40 [0.83-6.95]	0.108
High ($\geq$7 log_{10} DNA copies/g feces)	118	1.97 [0.80-4.85]	3.27 [1.09-9.83]	0.035
M. stadtmanae prevalence				
No	433	1.00 [reference]	1.00 [reference]	
Yes	39	1.20 [0.46-3.13]	1.14 [0.53-3.90]	0.483
Counts of archaeal species (log_{10} DNA copies/g feces)[e], median [range]				
M. smithii		1.05 [0.97-1.34]	1.10 [1.00-1.21]	0.047
M. stadtmanae		1.06 [0.92-1.22]	1.08 [0.94-1.25]	0.265

[a]GEE, generalized estimating equations.
[b]Sample size used for crude analysis, $N = 472$; OR = $e^{crude\beta}$ or $e^{adjusted\beta}$.
[c]Sample size for adjusted analysis, $N = 428$ and $N = 406$ for *M. smithii* and *M. stadtmanae,* respectively, due to missing values. Confounders in the final adjusted model for *M. smithii*: household size, place and mode of delivery, birth weight, dietary intake (total fiber intake, total percentage energy intake, percentage energy intake for fats and carbohydrates), antibiotic use, and physical activity; and for *M. stadtmanae*: household size, place and mode of delivery, birth weight, nutritional intake (total fiber intake, total percentage energy intake, percentage energy intake for fats and carbohydrates), physical activity, maternal level of education (low, middle, and high), and weight gain during pregnancy.
[d]Column represents *P*-values for the adjusted analysis.
[e]GEE analysis was done using archaea (*M. smithii* and/or *M. stadtmanae*) positive samples only.

promoting polysaccharide degradation and formation of SCFAs. It should, however, be noted that animal studies often show that increased fermentable fiber intake is associated with reduced body weight gain and/or adiposity, which may also depend on the animals' phenotype (29) likely due to differences in microbial fermentation capacity. A potential mechanism whereby methanogens may affect energy extraction and subsequently lead to overweight is through signaling of the G protein-coupled receptor Gpr41, for which SCFAs serve as ligands. Gpr41 expressed in the intestine and adipocytes stimulates the expression of the adipokine leptin and the intestinal peptide tyrosine–tyrosine (peptide-YY), which both influences energy metabolism and appetite level (17). Although, our results were in line with the above studies, other studies showed conflicting results compared with ours. Million et al. (30) found that the gut microbiota of humans with obesity is depleted in *M. smithii*. Two studies also reported that *M. smithii* was negatively correlated with BMI (19,31). Fernandes et al. (23) found that archaea presence was not associated with increased BMI. Armougom et al. (20) did not find a difference in the abundance of *M. smithii* in individuals with obesity compared with normal weight individuals. Differences in the methods and designs, such as techniques to detect methanogens, sample sizes, geographical settings, and dietary habits of the participants might all contribute to these different findings. Moreover, our study differs compared with most previous studies with respect to participants' weight status, which is in the normal range for the majority of subjects, and their young age.

We collected feces from the children at an age where stability of the gut microbiota is believed to be achieved and may be comparable to the adult microbiota. A number of studies revealed that the childhood microbiota has evolved into an adult-like configuration by the age of 2-3 years (32-34). Little is known whether the levels of archaea present in children are comparable to adults and studies have not yet been done on children above 3 years.

The large sample size and longitudinal design are major strengths of the present study. Questionnaires were repeatedly collected during the developmental stages of the children, yielding vast information on anthropometric data over time. The presence of detailed and prospective information on background factors enabled the adjustments of many confounders, including physical activity and diet. Results showed an independent association of archaea with weight outcomes, regardless of such confounding factors.

Our study also has some limitations. Repeated weight and height measurements in our study were parent-reported. A study by Scholtens et al. showed that this may lead to both an underestimation of the children's weight and an overestimation of their height, resulting in a lower BMI and lower prevalence of overweight (35). Another study showed the opposite, that is, an overestimation of weight implying more overweight in parent-reported information (36). A validation study using data from the KOALA Birth Cohort Study, found an underestimation of overweight was found with parent-reported data compared with data collected during home visits (37). Based on this, it is likely that anthropometric measurements by well-trained persons may lead to stronger associations between BMI and archaea. This may have resulted in an underestimation of the true associations.

Fecal samples of the children were collected only at one time point. We assumed that the colonization with archaea in the children is relatively constant over time. Indeed, previous studies

TABLE 5 GEE results showing association of different continuous outcomes (BMI, weight, and height *z*-scores) from 6 to 10 years of age with the prevalence of colonization and counts (log_{10} DNA copies/g feces) of archaea species in the gut microbiota at 6-7 years of age[a]

	n	Crude β [95% CI][b]	Adjusted β [95% CI][c]	*P*-value[d]
BMI *Z*-SCORES[e]				
M. smithii count levels				
None	103	0 [reference]	0 [reference]	
Low (<7 log_{10} DNA copies/g feces)	251	0.07 [−0.12 to 0.27]	0.10 [−0.09 to 0.29]	0.286
High (>7 log_{10} DNA copies/g feces)	118	0.09 [−0.14 to 0.32]	0.14 [−0.07 to 0.38]	0.194
M. stadtmanae prevalence				
No	433	0 [reference]	0 [reference]	
Yes	39	0.04 [−0.26 to 0.34]	0.15 [−0.17 to 0.46]	0.358
Counts of archaeal species (log_{10} DNA copies/g feces), median [range][f]				
M. smithii		0.00 [−0.02 to 0.03]	0.01 [−0.01 to 0.03]	0.436
M. stadtmanae		0.02 [−0.02 to 0.06]	0.03 [−0.01 to 0.07]	0.186
WEIGHT *Z*-SCORES				
M. smithii prevalence				
No	103	0 [reference]	0 [reference]	
Yes	369	0.13 [−0.06 to 0.31]	0.18 [0.00 to 0.36]	0.046
M. smithii count levels				
None	103	0 [reference]	0 [reference]	
Low (<7 log_{10} DNA copies/g feces)	251	0.12 [−0.08 to 0.32]	0.18 [−0.01 to 0.36]	0.071
High (>7 log_{10} DNA copies/g feces)	118	0.15 [−0.08 to 0.38]	0.20 [−0.02 to 0.40]	0.077
M. stadtmanae prevalence				
No	433	0 [reference]	0 [reference]	
Yes	39	0.16 [−0.15 to 0.48]	0.15 [−0.16 to 0.46]	0.335
Counts of archaeal species (log_{10} DNA copies/g feces), median [range][f]				
M. smithii		0.01 [−0.01 to 0.04]	0.01 [−0.01 to 0.04]	0.228
M. stadtmanae		0.04 [−0.01 to 0.08]	0.03 [−0.01 to 0.08]	0.107
HEIGHT *Z*-SCORES				
M. smithii prevalence				
No	103	0 [reference]	0 [reference]	
Yes	369	0.09 [−0.09 to 0.27]	0.13 [−0.05 to 0.31]	0.170
M. smithii count levels				
None	103	0 [reference]	0 [reference]	
Low (<7 log_{10} DNA copies/g feces)	251	0.10 [−0.10 to 0.29]	0.14 [−0.05 to 0.34]	0.134
High (>7 log_{10} DNA copies/g feces)	118	0.07 [−0.16 to 0.29]	0.07 [−0.13 to 0.29]	0.477
M. stadtmanae prevalence				
No	433	0 [reference]	0 [reference]	
Yes	39	0.15 [−0.16 to 0.46]	0.06 [−0.27 to 0.39]	0.726
Counts of archaeal species (log_{10} DNA copies/g feces), median [range]				
M. smithii		0.01 [−0.02 to 0.03]	0.01 [−0.02 to 0.03]	0.708
M. stadtmanae		0.03 [−0.01 to 0.08]	0.02 [−0.02 to 0.07]	0.369

[a]GEE, generalized estimating equations.
[b]Sample size used for crude analysis, $N = 472$.
[c]Sample size for adjusted analysis, $N = 428$ and $N = 406$ for *M. smithii* and *M. stadtmanae,* respectively, due to missing values. Confounders in the final adjusted model for *M. smithii*: household size, place and mode of delivery, birth weight, dietary intake (total fiber intake, total percentage energy intake, percentage energy intake for fats and carbohydrates), antibiotic use, and physical activity; and for *M. stadtmanae*: household size, place and mode of delivery, birth weight, nutritional intake (total fiber intake, total percentage energy intake, percentage energy intake for fats and carbohydrates), physical activity, maternal level of education (low, middle, and high), and weight gain during pregnancy.
[d]Column represents *P*-values for the adjusted analysis.
[e]Model with *M. smithii* prevalence as main determinant in association with BMI *z*-score not presented due to significant interaction with age.
[f]GEE analysis was done using archaea (*M. smithii* and/or *M. stadtmanae)* positive samples only.

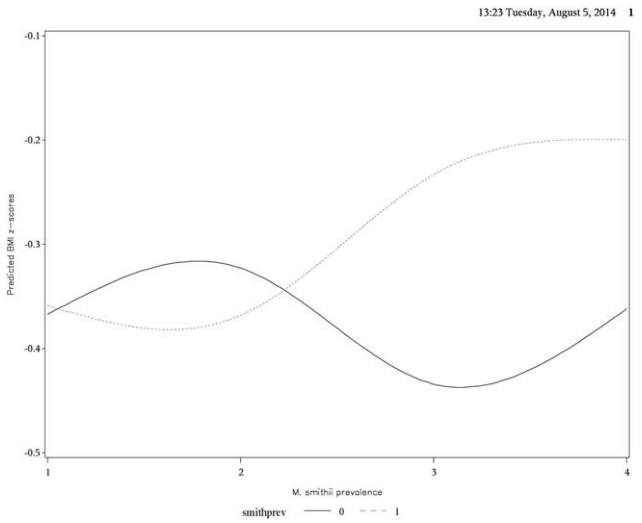

Figure 3 An interaction plot for the association of *M. smithii* prevalence and BMI *z*-scores across ages. The horizontal axis is the grouped ages at which different outcomes were measured. These are indexed in the variable time as follows (ages [mean ± SD] 6.2 ± 0.5, 6.8 ± 0.5, 7.8 ± 0.5, and 8.8 ± 0.5 years as time 1, time 2, time 3, and time 4, respectively); the vertical axis are the predicted BMI *z*-scores for each child at different time points obtained from the final GEE model; different line types represent curves for the two *M. smithii* prevalence groups (*smooth line* for uncolonized and *dotted line* for colonized). The smooth curves were obtained using spline interpolation.

have shown that the quantities of *M. smithii* in human feces remained constant over time (38). This trend of stability of *M. smithii* has also been reported over a 13-month period using fecal specimens from two individuals (39). Additionally, a comparative analysis of the genome of *M. smithii* and its transcriptome and metabolome in gnotobiotic mice in the absence and presence of *B. thetaiotaomicron* indicated that *M. smithii* survives in the intestinal tract of the gnotobiotic mice through different survival and colonization mechanisms despite the presence of its competitors for substrates (40). However, future studies are warranted in which fecal samples are collected at different time points of anthropometric measurements to accurately assess the change of archaea prevalence or counts in an individual over time. This could further establish changes in the archaeal microbiota due to different physiological, pathological, and iatrogenic (e.g., administration of certain antibiotics) conditions over time.

In conclusion, this study demonstrates that the presence as well as the higher counts of *M. smithii* in the gut of children at school age is associated with overweight, higher weight, and BMI *z*-scores from 6 to 10 years of age. This finding further supports the role of the methanogenic archaea in obesity after controlling for diet and physical activity which are the main factors associated with obesity. As such, manipulating the intestinal (archaeal) microbiota represents a potential strategy to apply together with dietary restrictions and exercise in the control of obesity. So far, little is known about the factors that determine archaea colonization in the gut. This indicates a pressing need for further research on these determinants of archaeal colonization which may be used to control colonization of the gut by archaea in the future.

Supporting Information on plasmid construction for positive controls (S1) and standard curves Figure S1(a) and (b) are available at www.onlinelibrary.wiley.com. O

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